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Multi-biomarker based Environmental Monitoring of Aquatic Ecosystems using *Catla catla* and *Cirrhinus mrigala* in Malwa Region of Punjab

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Abstract

Catla catla and *Cirrhinus mrigala* were collected from three districts in Malwa region of Punjab i.e. Muktsar, Bathinda and Ludhiana. Fulton's condition factor (K), protein content, qualitative analysis of proteins and genotoxicity was determined in fishes. Fulton's condition factor was higher in both fishes of Muktsar followed by Bathinda and Ludhiana. The lowest protein content was recorded in all tissues (liver, kidney, gills and muscles) of both fish species of Mukstar followed by Bathinda and Ludhiana with liver of both species and gills of *C. catla* showing non-significant variation whereas kidney as well as muscles of both species and gills of *C. mrigala* showing significant variation across three locations ($p<0.05$). SDS-PAGE analysis of liver revealed appearance of distinct protein bands such as 32 KDa, 38 KDa in *C. catla* and *C. mrigala* of both Mukstar and Bathinda as well as 120 KDa, 150 KDa and 200 KDa in *C. mrigala* of Bathinda only. Genotoxicity studies using the comet assay showed significant differences in tail lengths of liver cells, with Muktsar exhibiting the longest comet tails for both species. These findings suggest that *C. catla* and *C. mrigala* present in aquatic ecosystems of Muktsar were having elevated biomarker levels associated with environmental impact followed by Bathinda and Ludhiana which might be due to variations in pollutant load and other environmental stressors influencing aquatic ecosystem health.

Keywords: Fulton's condition factor, Environment, Genotoxicity, Protein content, SDS-PAGE

Introduction

Aquatic ecosystems are vulnerable to pollutants from domestic, urban and agricultural sources, including heavy metals and pesticides (Bashir *et al.,* 2020). These contaminants accumulate in aquatic animals and disrupt life cycles, posing long-term risks to aquatic organisms and ecosystems (Malik *et al.,* 2020). Exposure of aquatic animals to environmental contaminants such as pesticides, heavy metals etc. causes DNA damage in them (Kumar *et al.,* 2010). Heavy metals are persistent substances capable of bioaccumulating in organisms inhabiting aquatic and terrestrial ecosystems. These include elements such as Arsenic (As), Cadmium (Cd), Lead(Pb), Chromium (Cr), Cobalt (Co), Nickel (Ni), Mercury (Hg) etc. and previous literature reported that groundwater in Mukstar and Bathinda districts in the Malwa region of Punjab has been contaminated with various heavy metals (Singh *et al.,* 2021, Bangotra *et al.,* 2023). It poses serious health risks and causes increased incidence of cancer cases due to industrial waste, pesticides, heavy metals, radioactive substances such as uranium (Singh, 2024).

Fisheries and aquaculture are particularly vulnerable because contaminants like heavy metals, agrochemicals and industrial pollutants accumulate in water and sediments, adversely affecting aquatic organisms. Heavy metals and xenobiotics can induce oxidative stress, metabolic disturbances, impaired growth, reproductive toxicity and genotoxic effects in fish, making them valuable bioindicators for monitoring environmental quality. Biomarkers are sensitive tools for detecting early biological responses to environmental stress before severe ecological damage occurs. In biomonitoring studies, Fulton's condition factor (K) is widely used as a morphometric biomarker which act as an indicator of fish health and physiological status and variations in K may reflect the effects of pollution and other environmental stressors on fish populations. Besides this, proteins play essential roles in metabolic regulation, enzyme synthesis and physiological functioning; therefore, alterations in protein content may indicate contaminant-induced stress. Similarly, DNA damage, including strand breaks and adduct formation, reflects genotoxic effects of pollutants. The liver is particularly vulnerable because of its detoxification role (Mir *et al.,* 2014, Ardeshir *et al.,* 2017). The techniques such as comet assay and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) are widely used biomarkers for evaluating DNA damage and protein profile alterations in fish toxicological studies (Muhammad *et al.,* 2018).

Indian Major Carps such as *Catla catla* and *Cirrhinus mrigala* are economically important freshwater fish species and useful bioindicators of aquatic pollution. Previous studies have reported DNA damage and genotoxic responses in these species following heavy metal exposure (Kousar and Javed 2015, Mallesh *et al.,* 2015, Latif *et al.,* 2022). Punjab is an important aquaculture-producing state where carp culture contributes substantially to freshwater fish production. Carp culture involving *C. catla*, *C. mrigala* and *Labeo rohita* contribute 70 to 75% of the total freshwater fish production in Punjab (Bairwa *et al.,* 2023). The Malwa region districts, including Ludhiana, Bathinda and Muktsar are characterized by intensive agricultural activities and anthropogenic pressure which increase the possibility of aquatic contamination in these regions (Singh 2024).

Therefore, it was hypothesized that fish inhabiting aquatic ecosystems of selected Malwa districts may exhibit biomarker responses indicative of environmental stress. The present study was designed to evaluate protein content, DNA damage and SDS-PAGE protein profiling as multibiomarker approaches in *C. catla* and *C. mrigala* for monitoring aquatic ecosystem health.

Materials and Methods

Sampling locations

C. catla and *C. mrigala* were collected from three locations during February to March 2021 i.e. Fish pond of Organic Farming, PAU, Ludhiana; Fish market of Shri Muktsar Sahib (Morh road railway crossing) and Fish market of Bathinda (Market near railway station). Six individual fish (n = 6) of each fish species from each sampling location were collected and analyzed.

Morphometeric parameters of fishes

The morphometric parameters of fishes i.e. total length and body weight were observed and Fulton's condition factor (K) was calculated as follows:

$$K = \frac{\text{Body weight (g)} \times 100}{\text{Length (cm)^3}}$$

Autopsy of fish and preparation of tissue homogenates

Live fish were transported to the laboratory and anesthetized using clove oil until deep anesthesia was achieved, as indicated by loss of equilibrium and cessation of opercular movement. The fish were then euthanized and immediately dissected. Liver, kidney, gill, and muscle tissues were excised aseptically and processed for subsequent analyses.

Preparation of tissue homogenates

0.5g of each tissue (liver, kidney, gills, and muscles) was homogenised in 3 ml of phosphate buffer (PBS: 0.1 M, pH 7.4), and centrifuged at 3000 rpm for 10 minutes. After centrifugation, the supernatant was collected for protein estimation, SDS-page and genotoxicity studies.

Determination of protein content in tissues

The total soluble protein content in homogenates of various tissues was calculated using the method of Lowry *et al.* (1951). The amount of protein in different tissues was expressed as mg/g wet weight of tissue.

SDS-PAGE of liver tissue

The qualitative analysis of proteins was done by SDS-PAGE which was performed according to the method of Laemmli (1970) using 4% stacking gel and 12% resolving gel prepared in an electrophoresis apparatus. Protein samples (20 µg from pooled samples of liver tissue homogenates) were mixed with sample buffer and heated in a boiling water bath for 5–10 min to ensure complete denaturation. The prepared samples, along with a molecular weight marker were loaded into the wells of the gel and electrophoresis was carried out until adequate separation of protein bands was achieved. Following electrophoresis, the gel was stained with 0.5% Coomassie Brilliant Blue R-250 solution for 20–30 min and subsequently destained using a solution containing 20% methanol, 10% glacial acetic acid and 70% distilled water. The destaining solution was replaced several times until distinct protein bands appeared against a clear background. The molecular weight of protein bands was recorded in kilodaltons (KDa) after comparing with marker (10- 200 kilodalton bands) using software ImageJ (with Gel Analyzer plugin).

Genotoxicity studies in liver tissue

The genotoxic studies was conducted using Comet assay using 20 μl of liver tissue homogenate of fish by the method as described by Singh *et al.* (1988). Approximately 100 cells were taken for the analysis check the comet score under fluorescent microscope. The images of comets observed were taken under a fluorescent microscope at ×200 and tail length of comets (in μm) was recorded using Open Comet-ImageJ plugin software.

Statistical Analysis

The data was presented as Mean± Standard Error. Statistical package for Social sciences (SPSS) version 16.0 software was used to determine statistical differences using one-way analysis of variance (ANOVA) and Duncan's Post-hoc test.

Results and Discussion

Morphometric parameters

The Fulton's condition factor (K) was observed to be non-significantly maximum for *C.catla* of Muktsar (2.72±0.24) followed by Bathinda (2.69±0.23) and Ludhiana (2.66±0.23) (Fig. 1). Similarly, non-significantly highest Fulton index was recorded in *C. mrigala* of Muktsar (2.62±0.22) followed by Bathinda (2.60±0.22) and Ludhiana

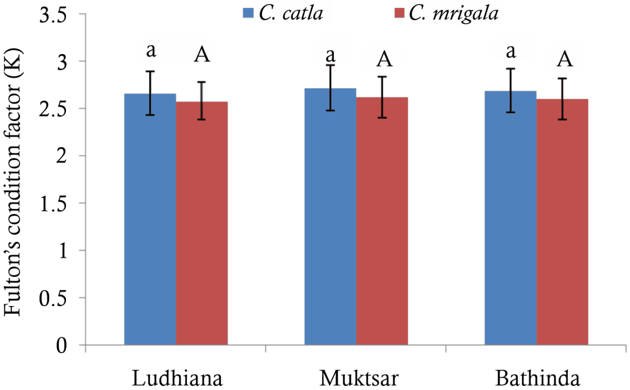

Fig. 1 Fulton's condition factor of *Catla catla* and *Cirrhinus mrigala* at three locations. Values are expressed as Mean ± SE. Values with similar superscript (a and A) above bars are non-significantly different (p<0.05)

(2.58±0.20). Fulton's condition factor have been used to evaluate the stress of caused by environmental contaminants such as heavy metal bioaccumulation in fish (Ju *et al.,* 2017) and the fishes from polluted water showed lower Fulton's condition factor as compared to non-polluted water (Kerambrun *et al.,* 2013). Fulton condition factor more than one at all three locations indicated good health condition of fishes and less variations in environmental conditions of the aquatic ecosystems at the three locations may be responsible for non-significant variation in Fulton condition factor among the locations.

Protein content

The protein content in liver of *C. catla* among three locations varied non-significantly from each other and it was found to be lowest in Muktsar (35.59±2.49 mg g^{-1} wet wt) followed by Bathinda (36.26±1.20 mg g^{-1} wet wt) and Ludhiana (36.33±1.19 mg g^{-1} wet wt) (Fig. 2). The protein content for species *C. mrigala* among three locations also varied non-significantly with each other with lowest total protein content was recorded in Bathinda (38.40±1.51 mg g^{-1} wet) followed by Muktsar (38.40±0.40 mg g^{-1} wet wt) and Ludhiana(38.62±1.53 mg g^{-1} wet wt).

The significantly low total protein content was observed in the kidney of *C. catla* of Muktsar (13.42±0.45 mg g^{-1} wet wt) and Bathinda (14.69±0.95 mg g^{-1} wet wt) as compared to Ludhiana(25.51±3.21 mg g^{-1} wet wt).The lowest

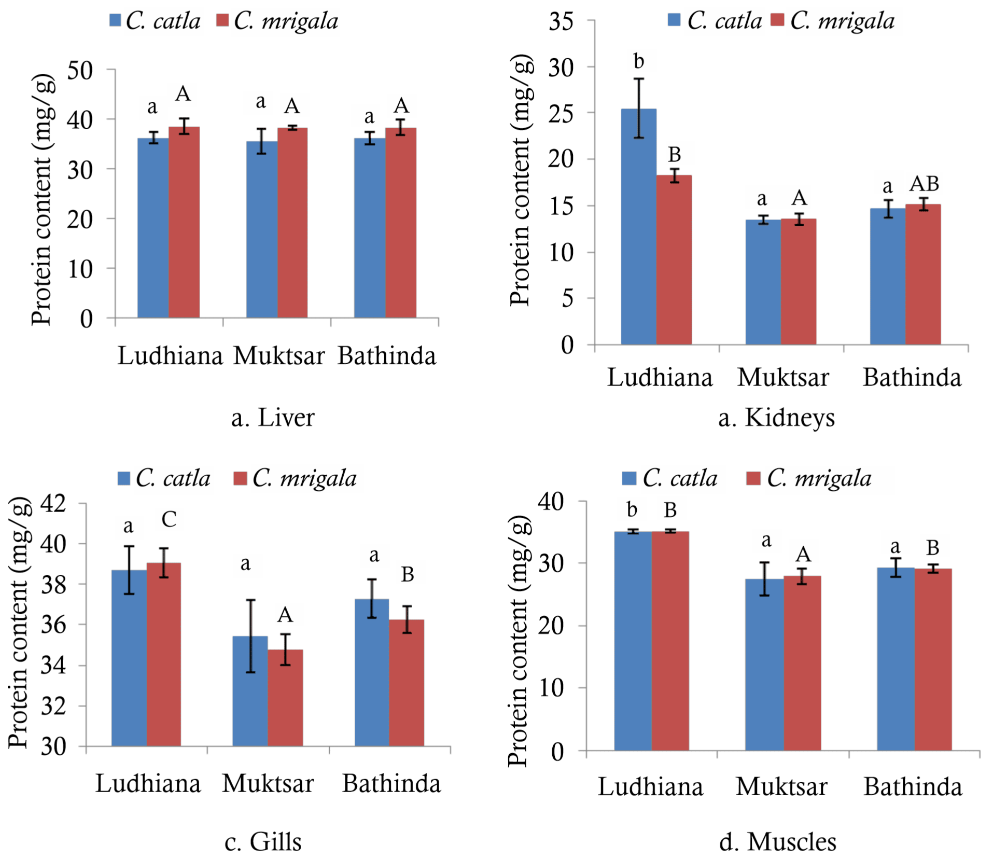

Fig. 2 Protein content (mg/g wet weight) in different tissues of *Catla catla* and *Cirrhinus mrigala* at three locations. Values are expressed as Mean ± SE. Values with different superscript (a-b and A-C) above bars are significantly different ($p<0.05$)

protein content in kidney of *C. mrigala* of Muktsar (13.53±0.58 mg/g wet wt) varied non-significantly with Bathinda (15.13±0.69 mg/g wet wt) and significantly with Ludhiana (18.24±0.76 mg/g wet wt).

The lowest protein content was observed in gills of *C. catla* of Muktsar (35.42±1.79 mg/g wet wt) followed by Bathinda (37.28±0.94 mg/g wet wt) and Ludhiana (38.71±1.19 mg/g wet wt) and the protein content varied non-significantly at three locations. The total protein content in gills of *C. mrigala* was found to be significantly less in Muktsar (34.78±0.76 mg/g wet wt) and Bathinda (36.26±0.67 mg/g wet wt) as compared to Ludhiana (39.06±0.70 mg/g wet wt).

The significantly less total protein content in the muscles of *C. catla* was found in Muktsar (27.49±2.64 mg/g wet wt) and Bathinda (29.38±1.47 mg/g wet wt) as compared to Ludhiana (35.19±0.32 mg/g wet wt). Similarly, lowest protein content in muscles of *C. mrigala* was found in Muktsar (27.98±1.25 mg/g wet wt) followed by Bathinda (29.15±0.63 mg/g wet wt) and Ludhiana (35.23±0.25 mg/g wet wt). The protein content in muscles of *C. mrigala* of Muktsar varied significantly from Bathinda and Ludhiana and there was non-significant variation in protein content of muscles of *C. mrigala* from Ludhiana and Bathinda.

The findings of the present study showed decrease in total protein content in various tissues of both fishes of Muktsar and Bathinda as compared to Ludhiana and this might be due to damage at cellular or molecular level as well as negatively affecting the process of protein synthesis caused by contaminants such as pesticides, heavy metals etc. The groundwater of Muktsar was found to be contaminated with heavy metals (Hg, As, Pb) and pesticide residues (Glyphosate) (Bangotra *et al.,* 2023). Similarly, the heavy metals such as (As, Cr, Pb) and the pesticides like chlorpyrifos have been found in the groundwater of Bathinda (Singh *et al.,* 2021). The energy requirement of the carps in the Muktsar and Bathinda districts might been increased as the result of defense mechanism against contaminants so there could be possible breakdown of proteins in order to maintain normal energy levels in the fish. The reduced protein content was observed in different organs such as brain, muscle, gills, liver, kidney and heart caused due to environmental pollutant such as carbosulfan (pesticide) in *Clarias batrachus* (Thoker, 2015). The toxicity caused by heavy metals such as Cd resulted in decrease in protein fractions in different tissues in *C.batrachus* (Khan *et al.,* 2001). The protein content in muscles of catfish decreased due to pollutants such as heavy metals (Habib, 2013).

SDS-PAGE

SDS-PAGE of liver tissue homogenates of *C. catla* and *C. mrigala* of three different locations i.e. Muktsar, Ludhiana, Bathinda showed appearance of some distinct protein bands at different locations which may serve as a biomarker for monitoring of environmental stress. The liver of *C. catla* of Muktsar showed protein bands with molecular weight of 10 KDa, 15 KDa, 24 KDa, 30 KDa, 32 KDa, 38 KDa, 50 KDa and 70 KDa (Fig. 3). The liver of *C. catla* of Bathinda showed presence of 10 KDa, 15 KDa, 24 KDa, 50 KDa, 70 KDa protein bands. The liver of *C. catla* of Ludhiana showed proteins of bands 10 KDa, 15 KDa, 24 KDa, 30 KDa, 50 KDa, 70 KDa molecular weight. The *C. mrigala* of Muktsar showed the presence of 10 KDa, 15 KDa, 24 KDa, 30 KDa, 32 KDa, 38 KDa, 50 KDa, 70 KDa, 120 KDa and 150 KDa protein bands. The liver of *C.mrigala* of Bathinda showed occurrence of 15 KDa, 20 KDa, 30 KDa, 38 KDa, 50 KDa, 70 KDa, 120 KDa, 150 KDa, 200 KDa protein .The liver of *C.mrigala* of Ludhiana showed the presence of 10 KDa, 15 KDa, 24 KDa, 50 KDa, 70 KDa protein bands.

C. catla of Muktsar showed bands of 32 KDa and 38 KDa molecular weight and *C.mrigala* of both Muktsar and Bathinda showed bands of 30 KDa, 32 KDa, 38 KDa, 120 KDa, 150 KDa and 200 KDa molecular weight which were not present in Ludhiana indicating that these proteins may serve as indicators for environmental monitoring of aquatic ecosystems in these areas. Mathew *et al.,* (2023) reported that SDS-PAGE analysis of different tissues of *Cyprinus carpio* showed expression of additional protein bands in response to exposure to heavy metals such as lead and mercury. Raj and Joseph (2017) reported increased stress protein bands in the brain,gills and liver of *Oreochromis mossambicus* serving as biochemical indicators of pesticide (acetamiprid) pollution. Sharma and Jain (2008) found appearance of additional bands of 30.2 KDa, 64.6 KDa and 77.6

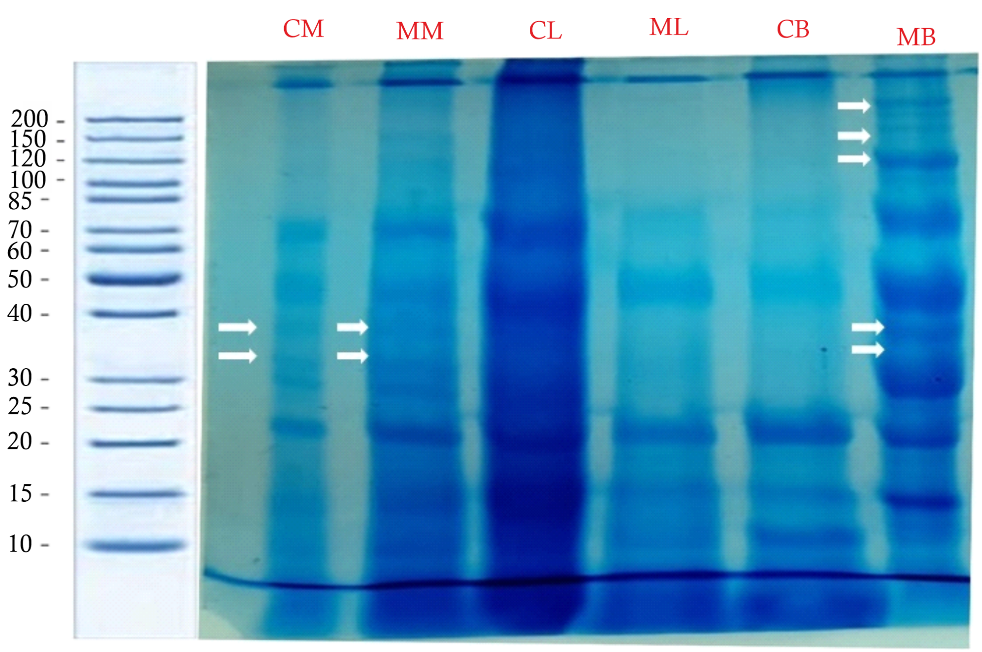

Fig. 3 SDS-PAGE of liver tissue homegenate of carps, CM, CL and CB represent *Catla catla* and MM, ML and MB represent *Cirrhinus mrigala* of Muktsar, Ludhiana and Bathinda, respectively

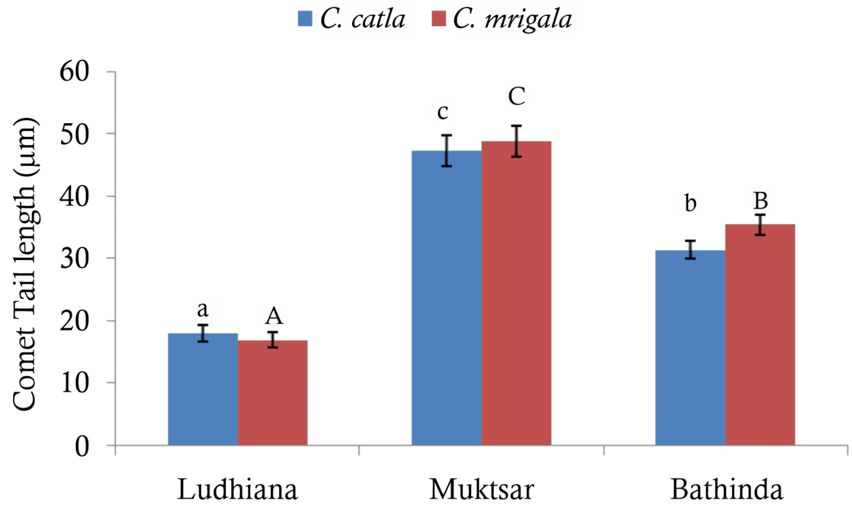

Fig. 4 Comet tail length (µm) in liver cells of *Catla catla* and *Cirrhinus mrigala* at three locations

KDa in the liver tissue of fish, *L. rohita* after long term exposure to different pesticides such as endosulphan, malathion and chlorpyrifos. However, consistent appearance of certain protein bands across locations, such as 10 KDa, 15 KDa, and 70 KDa, in both *C. catla* and *C. mrigala* may represent constitutive proteins, common to both species and reflecting basic metabolic functions.

Genotoxicity studies

The genotoxicity studies indicated that a significant difference was observed in tail length of comets of *C. catla* among three locations with maximum value recorded for Muktsar (47.25±1.48 µm) followed by Bathinda (31.37± 2.07 µm) and Ludhiana (17.99±1.28 µm) (Fig.4 , Fig. 5 (i-iii)). Similarly, significant difference in comet tail length of *C.mrigala* was found among three locations with highest comet length in liver cells of *C.mrigala* observed in Muktsar (48.80±2.02 µm) followed by Bathinda (35.42±1.55 µm)and Ludhiana (16.93±1.62 µm) (Fig. 6(i-iii)).

The genotoxicity studies in *C.calta* and *C. mrigala* indicate notable location-based variations

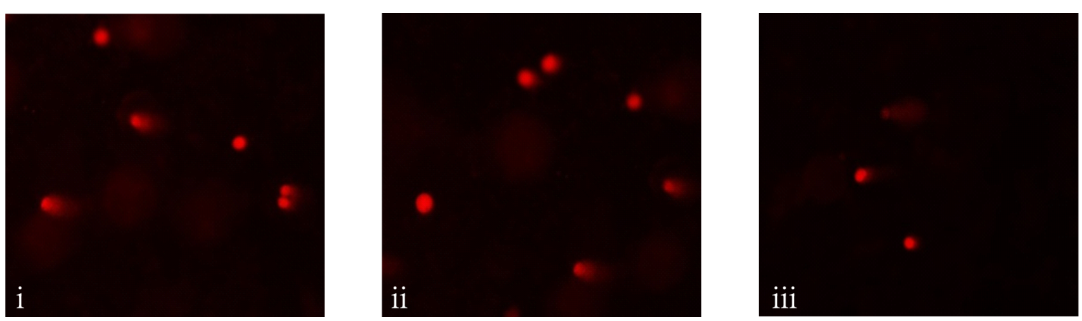

Fig. 5 Comet images of liver cells of *Catla catla* of three locations: i) Muktsar, ii) Bathinda and iii) Ludhiana

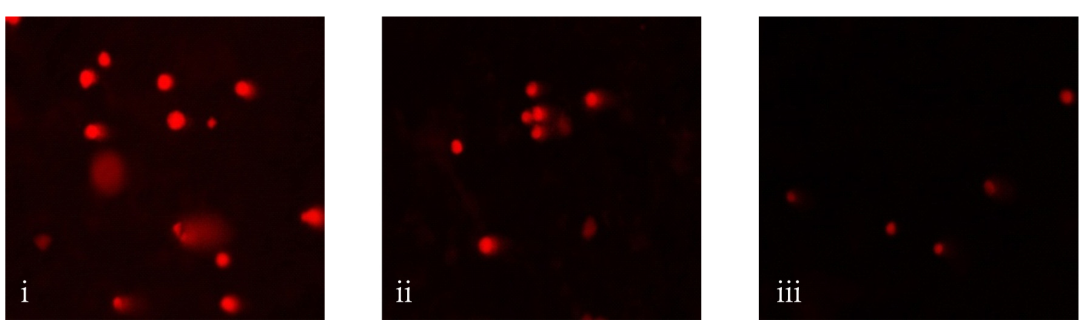

Fig. 6 Comet images of liver cells of *Cirrhinus mrigala* of three locations: i) Muktsar, ii) Bathinda and iii) Ludhiana

in comet tail lengths of liver cells, suggesting differential levels of DNA damage among the studied sites. These findings highlight the potential impact of environmental or ecological factors at each location that may contribute to the observed genotoxic effects. The comet assay showed that prolonged exposure to water pollutants resulted in excessive breakage in DNA strand (Ternjej *et al.,* 2010). The heavy metals such as Cr caused genotoxicity as well as endocrine disruption in freshwater fishes (Velma *et al.,* 2009). The presence of some metals in the water could be one of the mechanisms that caused genetic harm in the fish. Teles *et al.,* (2005) found genotoxic effects in European eel (*Anguilla anguilla*) after being exposed to Cr for a short period of time. The genotoxic effects of heavy metals such as Cu, Zn, Fe, Pb and Cd was observed in grass carp, *Ctenopharyngodon idella* in river Ili of Kazakhstan (Tlenshieva *et al.,* 2022). The exposure of fish to samples from agricultural areas induced significantly higher number of genotoxic effects in fish such as *Cichlasoma paranaense* (Francisco *et al.,* 2023). Similarly, Dutta *et al.* (2024) observed more genotoxic impact on the resident fish, *Pethia conchonius* present in the contaminated sites due to prevalence of the insecticides such as imidacloprid, chlorpyrifos, bifenethrin, cypermethrin, difenthiuron and acetamiprid.The herbicide Roundup caused showed genotoxic effects in blood, gill, and liver cells of *Piaractus mesopotamicus* as revealed by comet assay analysis.

Conclusion

The present study demonstrated significant variations in biomarker responses such as alterations in protein content, DNA damage, SDS-PAGE protein profiles among fish collected from different locations of the Malwa region of Punjab which suggest varying levels of environmental stress among the studied locations and highlight the usefulness of a multi-biomarker approach for assessing fish health and monitoring the effects of environmental pollutants in aquatic ecosystems.

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